Basic Studies towards Elucidation of Heartwood Formation Mechanisms

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Preliminary

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Introduction

Heartwood, which is the colored tissue in inner trunks of trees, is composed of only dead cells and supports the trunk physically. Heartwood formation is a metabolic event specific to woody plants, which does not occur in herbaceous plants, and elucidation of the heartwood formation mechanisms can be a clue to help us understand mechanisms for woody-plant specific metabolisms.

First, studies of gene function require a system for transformation and regeneration of target plants. In *Robinia pseudoacacia* (black locust), heartwood formation starts a few years after the cell division[1], which is much earlier than other trees, indicating that this species is a good model plant for studies of heartwood formation mechanisms. Recently, Igasaki et al. reported for the first time *Agrobacterium tumefaciens*-mediated transformation of *R. pseudoacacia* using shoots from a single mature seed[2]. However, regeneration conditions for shoots from mature *Robinia* tree was studied in this research, because mature seeds have a high level of genetic diversity.

Second, an important characteristic of heartwood formation is that the metabolic event is accompanied by the biosynthesis and deposition of significant amounts of secondary metabolites, so-called heartwood substances like lignans, norlignans, and flavonoids[3]. These compounds, especially lignans and norlignans, are biosynthesized through the cinnamate/monolignol pathway, where caffeoyl CoA *O*-methyltransferase (CoAOMT) is a key enzyme. *Carthamus tinctorius* (safflower) produces significant amounts of lignans during seed maturation[4], and several *CoAOMT* cDNAs have been cloned from the cDNA library prepared from the maturing seeds[5]. Herein, as the first step to understand the functions of *CtCoAOMT*, *CtCoAOMT* cDNAs were expressed in *E. coli.*

1. Regeneration of *Robinia pseudoacacia*

1.1 Experimental

Young shoots from mature *R. pseudoacacia* trees were cut into 5–6 cm pieces, and sterilized shoot explants were placed on MS basal medium including 0.01 mg/l 2,4-dichlorophenoxyacetic acid and 5 mg/l kinetin (MSB5DK)[6] or MS basal medium without plant growth regulators (MSB5S)[2]. Axillary shoots or calli were formed from the shoot explants. The calli formed were subcultured into MS basal medium with plant growth regulators to induce adventitious shoots.

Explants from the axillary and adventitious shoots were placed on MS basal medium containing 0.05 mg/l 2,4-dichlorophenoxyacetic acid, 2.5 mg/l 6-benzyladenine, and 2.5 mg/l *trans*-zeatin (MSB5DBZ)[2] to induce shoot regeneration.

Root formation from the regeneration shoots was induced on 1/2 MS basal medium containing indolebutyric acid (IBA). Rooted plantlets were gradually acclimatized to ambient relative humidity and transferred to the outside nursery.

1.2 Results and Discussion

Axillary shoots were formed from the shoot explants. No difference in shoot generation between MSB5S[2] and MSB5DK[6] media was observed. Some of the explants also resulted in callus formation. However, the shooting efficiency of adventitious shoots from callus was low under the conditions employed. The axillary and adventitious shoots thus obtained were used as explant sources for further experiments. When stem explants from the aseptic shoots were cultured on MSB5DBZ medium, shoot regeneration efficiency was 87%, while 8% of petiole explants produced shoots, but no shoot formation occurred from leaflet explants on the same medium.

Root formation of the regenerated shoots was done on 1/2 MS basal medium containing IBA. Although incubation for one month hardly resulted in root formation, continuing the incubation for two to four months by subculturing every one month led to root formation at the high efficiency (about 80%). The rooted plantlets were acclimatized and transplanted to the outside nursery. Almost all transplants survived and exhibited active growth.

2. Expression and characterization of recombinant *Carthamus* CoAOMT1 and 2

2.1 Experimental

Polymerase chain reaction (PCR) was employed to introduce the *Nde* I site at the 5'-end and *Not* I site at the 3'-

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end of the coding sequence of each *Carthamus CoAOMT* cDNA (*CtCoAOMT1* and *CtCoAOMT2*) with two designed primers. Each PCR product was first cloned into a pCR2.1-TOPO vector (Invitrogen). After confirmed to have no PCR errors, *CtCoAOMT1* and *CtCoAOMT2* cloned in pCR2.1-TOPO vector were digested with *Nde* I and *Not* I and ligated to pET-23a plasmid (Novagen). Each expression plasmid DNA was transformed into *E. coli* JM109 compitent cells and a single clone picked for plasmid DNA preparation. Each construct was transformed into *E. coli* BL21 (DE3) compitent cells for expression. The growth and induction of bacterial cells with isopropyl β-D-thiogalactoside were performed according to the manufacture's protocols (Novagen). After harvesting by centrifugation, the cell pellet containing CoAOMT proteins was processed to affinity-purify the CoAOMT proteins using His·Bind Resin (Novagen). Purified CoAOMT proteins were used for enzyme assay, and the products of these reactions were analyzed by GC-MS.

2.2 Results and Discussion

The purified recombinant CtCoAOMT1 was tested in vitro for their activity towards putative phenylpropanoid substrates : caffeoyl CoA (CaCoA), 5-hydroxyferuloyl CoA (5-HFCoA), caffeic acid (CA), and 5-hydroxyferulic acid (5-HFA). The recombinant *Ct*CoAOMT1 catalyzed *O*-methylation of 3 and 5-hydroxyl groups of CaCoA and 5-HFCoA, respectively, whereas both CA and 5-HFA did not serve as substrates. The recombinant *Ct*CoAOMT2 showed the same substrate specificity, indicating clearly that these recombinant proteins are caffeoyl CoA *O*-methyltransferases.

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